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Evolutionary conservation of the endogenous cardiac regenerative response

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Submitted
Abstract

In contrast to lower vertebrates, the mammalian heart has a very limited regenerative capacity. Cardiomyocytes, lost upon ischemia, are replaced by fibroblasts. Although the human heart is able to form new cardiomyocytes throughout its lifespan, the efficiency of such phenomenon is not enough to recover a significant myocardial mass after myocardial infarction. In contrast, injured zebrafish hearts regenerate by epicardial activation and initiation of myocardial proliferation. We investigated these processes in a mouse myocardial infarction model. Subsequent to the loss of epicardial cells overlying the ischemic region, a new epicardial layer was formed in two days. In these epicardial cells embryonic epicardial genes, such as Wilms' tumor 1, Tbx18 and Raldh1 were transiently expressed. Concomitant with this activation, subepicardial mesenchyme was formed and proliferation was initiated in the epicardium. As it has been described for embryonic development, the subepicardial mesenchyme formed during this short recovery period, differentiates into cardiomyocytes, cardiac fibroblasts and coronary endothelial cells. Thus, as reported for lower vertebrates, mice initiate an endogenous epicardial-dependent regenerative response to injury. This response, however, contributes only to a limited amount of cardiomyocytes and is insufficient to regenerate damaged cardiac muscle heart. Our molecular analyses provide the base required for subsequent investigations on the regeneration of the mammalian heart.
Introduction

The limited regenerative capacity of the human heart in response to injury leads to the severest forms of morbidity worldwide (http://www.who.int/mediacentre/factsheets/fs317/en/index.html). Understanding the mechanisms underlying this vulnerability of the heart has become a fertile topic for research and debate for the last decades. Subsequent to ischemic damage, the contractility of the heart is diminished due to loss of cardiomyocytes and replacement by non-contractile fibrotic cells. New cardiomyocytes, that integrate and function within the damaged heart, are needed to restore its function.

The ability to create new cardiomyocytes from undifferentiated cells is a challenging inroad to heal the damaged heart. Application of stem cells in cardiac regenerative medicine has, however, faced serious hurdles regarding the integration of newly formed cardiomyocytes into the damaged myocardium to restore cardiac function.1 Therefore, stimulation of the endogenous regenerative capacity seems an attractive alternative to this stem cell approach. Encouraging insights for this latter approach came from the unequivocal demonstration of endogenous regenerative capacity of the human heart.2 Although these insights showed that the regenerative capacity of the heart is sufficient for normal life, it is, for unknown reasons, insufficient to repair cardiac damage subsequent to cardiac infarction.

An essential prerequisite to stimulate the endogenous regenerative capacity of the heart is the understanding of the mechanisms underlying this regenerative process. The cellular origin of the regenerative capacity of the heart is largely unknown. Genetic labeling and tracing of adult endocardial cells in mice showed that upon cardiac damage endocardial cells transform into fibroblasts by a process called Endocardial-to-Mesenchymal Transformation, but not into cardiomyocytes.3 Genetic lineage tracing of cardiomyocytes in the mouse heart showed that a limited number of cardiomyocytes is formed upon damage, but that these cardiomyocytes do not originate from existing myocardium.4 In zebrafish, however, proliferation of existing cardiomyocytes was found to regenerate the myocardium upon amputation of the cardiac apex.5,6 Nonetheless, activation of epicardial cells by re-expression of so-called embryonic genes was also shown to be crucial in this regenerative response.7

Recently, it was reported that also adult mammalian epicardium expresses embryonic epicardial genes in response to injury.8-11 To investigate the role of the epicardium in the endogenous regenerative capacity of the mammalian heart, we induced a myocardial infarction (MI) in epicardial lineage reporter mice and analyzed the cellular and molecular responses at various time points post-MI. We observed that epicardial cells over laying the ischemic area had disappeared one day post-MI. The epicardial cells flanking the ischemic area (border zone) transiently re-expressed embryonic epicardial markers genes, like Wt1, Tbx18, Raldh1 and 2, and grew, by proliferation over the infarcted area. In this newly formed epicardium, also genes important for Epithelial-to-Mesenchymal-Transformation (EMT) were expressed and a subepicardial mesenchymal layer was formed. Lineage analysis
further demonstrated that the formed mesenchymal cells contributed to the infarcted area, and differentiated not only into myofibroblasts and cells of the coronaries, but also into cardiomyocytes. The differentiation into the different cell lineages is suggested to be regulated by the cooperative interaction between BMP- and FGF-signaling.

Materials and methods

Animals
All experimental procedures complied with national and institutional guidelines. To generate the Wt1 (BAC)-IRES-EGFP-Cre transgenic line, further referred to as Wt1 lineage reporter, the BAC clone RP23-266M16, ranging from +127kbp upstream to -11.5kbp downstream relative to the transcription start site of Wt1, was modified by inserting an IRES/EGFP-Cre cassette 17bp downstream of the translation stop site of the Wt1 gene. The R26R transgenic mouse line has previously been described. All mice were bred on a FVB background (Harlan, Indianapolis, Ind). As controls wild-type littermates were used.

Surgical procedure
Mice were anesthetized through intraperitoneal injection, using a cocktail of Fentanyl (0.07 mg/kg), Dormicum (7 mg/kg), Dex-Domitor (0.35 mg/kg) and Atropine (0.05 mg/kg). Myocardial infarction (MI) was induced by ligation of the left anterior descending (LAD) coronary artery at 8-12 weeks of age (20 gram body weight) as previously described. Briefly, the anesthetized mouse was orally intubated using a 20-gauge intravenous catheter with a blunt end, and mechanically ventilated using a rodent ventilator (MiniVent, Harvard Biosciences). The mouse was placed on a heating pad to maintain its body temperature. A left thoracotomy was performed at the third intercostal space and muscles were dissected. The LAD was permanently ligated using a 9-0 unabsorbable ethilon suture. After verification of change of color, and kinesis of the apex and anterior-lateral wall to ensure coronary occlusion, the thorax was closed in layers. The anesthesia was antagonized using Antisedan (2.5 mg/kg) and Flumazenil (Anexate) (0.5 mg/kg). After detubation, mice were kept warm until fully recovered. At least three mice were sacrificed at 1, 2, 3, 7, 14 days, 1 month, and 3 months after MI. 4 Hours before sacrifice mice were injected intraperitoneally with a solution of 10mg/ml 5’-Bromo-2’-Deoxyuridine (BrdU; Sigma) in physiological salt (50 mg/kg). After sacrifice the hearts were flushed with phosphate-buffered saline (PBS) and dissected. One half was fixed overnight in freshly dissolved 4%(w/v) paraformaldehyde (PFA) in PBS, the other half was snap-frozen in liquid nitrogen and stored at −80°C.

In situ hybridization and immunohistochemistry
Fixed hearts were embedded in paraplast and sectioned at 7μm for immunohistochemistry or at 10μm for in situ hybridization. Sections were deparaffinized and rehydrated in a graded alcohol series. In situ hybridization was performed as previously described. Cardiac Troponin
Endogenous epicardial regenerative response
I (cTnI), Raldh1, Raldh2, Wt1, Tbx18, Snai1, Periostin and Fstl1 specific probes were used. Immunohistochemistry was performed as previously described.\textsuperscript{16} In short, sections were boiled for 5 minutes in antigen unmasking solution (H3300, Vector), the signal was amplified with tyramide signal amplification (TSA NEL702, Perkin Elmer) and background signals were reduced using heart extract powder. The following primary antibodies were used: anti- Troponin I (TnI) (1:500 MiliPore), anti-Wt1 (1:200 Santa Cruz), anti-PDGFR\textalpha{} (1:200 Santa Cruz), anti-CD34 (1:200 BD bioscience), anti-cKit (1:200 Southern biotech), anti-α-sma (1:1000 Sigma) anti-BrdU (1:600 Becton Dickinson), anti-P-Smad1/5/8, anti-P-Erk1/2 (1:200 Cell Signaling Technology), anti-PECAM (1:100 Santa Cruz, and β-Galactosidase (1:500 MP Biomedicals(Cappel)). Alexa488 or Alexa568 conjugated goat-anti-rabbit and goat-anti-mouse antibodies (1:200 Molecular Probes) were used as secondary antibodies. Nuclei were visualized using Topro3 (1:500 Molecular Probes).

Images were recorded using a confocal laser scanning microscope (Leica SPE) for immunohistochemical stains and an AxioPhot microscope (Zeiss) for in-situ-hybridization stainings.

\textbf{Results}

\textbf{Lineage analysis of the epicardium in response to injury}
To analyze the epicardial response to injury, Wt1 lineage reporter mice were crossed with R26R reporter mice and the expression of β-Gal after a myocardial infarction was studied. In the hearts of healthy Wt1-lineage mice, β-Gal is expressed in the entire epicardium, endothelial cells of the coronaries and in intermyocardial cells, most probably cardiac fibroblasts. In these hearts no β-Gal positive myocytes were observed (figure 1). One day post-MI, the epicardium overlying the infarcted area is disrupted, and only a few β-Gal positive cells were detected, whereas β-Gal was found in the epicardium overlying the uninjured myocardium and border zone of the infarct, like in the controls (figure 1). Three days post-MI, the epicardial layer, which expressed β-Gal, was restored covering the entire infarcted area. Underneath this epicardial layer a β-Gal-expressing mesenchymal layer had formed, suggesting an epicardial origin. At seven days post-MI the space between the epicardium and myocardium was extended. Part of the β-Gal positive subepicardial cells was found to express α-sma, indicating their differentiation into myofibroblasts. One month post-MI, the mesenchymal layer was still present in the border zone but had disappeared form the infarcted area. The β-Gal expressing epicardial cells had not disappeared but still covered the entire infarcted area. Within the infarcted area many β-Gal positive cells were identified. To further analyze the phenotype of these cells, double immunofluorescent staining was performed using PECAM to detect endothelial cells and cTnI to detect myocardial cells. Within the infarcted area β-Gal and PECAM double positive cells were found indicating that epicardial-derived cells have differentiated into endothelial cells. Interestingly, also a small number of β-Gal and cTnI double positive cells were identified in the infarcted area of the heart. Most β-Gal
**Figure 1. Post ischemic lineage analysis of Wt1(Bac)IRF-EGFP-Cre**

In control Wt1(Bac)IRF-EGFP-Cre X R26R hearts β-Gal is detected in the entire epicardium covering the ventricles, atria and atrioventricular junction (a,b). βGal is also detected in cells of the coronaries and in inter-myocardial cells (a,a'). One day post-MI, no epicardial β-Gal-expressing cells were observed in the epicardium covering the infarcted area of the heart (c). In the epicardium and coronaries of the border zone β-Gal was still present (d). Three days post-MI, β-Gal-expressing epicardial cells cover the infarcted area again (e). In the border zone a subepicardial β-Gal-expressing layer of cells appears (f,g). In the uninjured areas of the same heart β-Gal expression was comparable to the control situation (h). One week post-MI the number of subepicardial β-Gal-expressing cells has increased (double arrow j), and α-sma and β-Gal double positive cells are present (k) within the subepicardial layer, while in the uninjured area of the heart the epicardium directly overlays the myocardium (l). One month post-MI β-Gal-expressing cells are present in the infarcted area and the epicardium covering the infarcted area (m,n), β-Gal-positive cells co-express cTnl (m) and PECAM (n). Three month post-MI a few β-Gal- and cTnl-expressing cells are detected in the infarcted area of the heart (o,p).
and cTnI double positive cells were detected at three months post-MI. β-Gal and cTnI double positive cells were never observed within the healthy portion of the myocardium at any of the time points evaluated.

Taken together β-Gal expressing epicardial cells disappear from the infarcted area of the heart directly upon ischemia. The epicardial layer is quickly restored as from three days post-MI onwards β-Gal expressing cells are again found to cover the infarction. Upon restoring the epicardium, the subepicardial layer starts to expand and becomes populated by epicardial-derived mesenchyme. From one month post-MI onwards, lineage-positive cells are found in the infarcted area. Phenotyping these cells revealed that they had differentiated into myofibroblasts, endothelial cells and cardiomyocytes. Although at three month post-MI the population of lineage positive cardiomyocytes had expanded, its contribution to the infarcted area was limited.

These processes are remarkably similar to the formation of epicardium, and the accompanying cellular migration and mesenchyme formation during cardiac development. Therefore, we systematically investigated the expression of markers for embryonic epicardium, and mesenchyme formation, as well as proliferation.

**Adult epicardium expresses “embryonic epicardial genes”**

During development, the transcription factors T-box 18 (Tbx18), Wilms’ tumor 1 (Wt1), and the retinoic acid metabolizing enzymes Raldh1 and 2 are expressed throughout the entire epicardium and become down-regulated at the end of gestation. The patterns of expression of these genes are associated with the developmental functions of the epicardium, like Epithelial-to-Mesenchymal-Transformation (EMT), formation of coronary vessels, and myocardial growth and differentiation. In the adult heart, the patterns of expression of these marker genes have not been described in detail, which is, however, essential as these genes are reported to play a crucial role in the processes accompanying MI.

Wt1 and Raldh1 and 2 were detected in the entire epicardium covering the atria, in the epicardial cells overlying the atrioventricular junction and the apex (figure 2). None of these genes were detected in the epicardium covering the ventricles. Tbx18 was expressed only in a minority of epicardial cells covering the atria and atrio-ventricular junction (figure 2).

Because the epicardium has been suggested to be a source of cardiac progenitors during development, we determined the pattern of expression of the progenitor markers Platelet Derived Growth Factor α (PDGFRα), CD34 and cKit by immuno-histochemistry (figure 2). PDGFRα, CD34 and cKit were detected in the epicardium covering the atria, atrioventricular sulcus and apex. PDGFRα and CD34 were also found to be expressed in a subset of mesenchymal cells in the atrioventricular sulcus. In line with these observations, the atrioventricular sulcus and apex were described as stem cell niches in the adult heart. Activation of embryonic epicardial genes upon injury

To investigate the changes in the spatio-temporal pattern of expression of Wt1, Tbx18, Raldh1, and 2 upon a myocardial infarction, hearts were collected at different time points
Figure 2. Expression of embryonic epicardial and progenitor markers in the adult mice heart

Panel a shows the pattern of expression of cTnI mRNA to identify cardiomyocytes in the adult mice heart. In this panel the boxes indicate the left atrium (LA) (1), atrioventricular sulcus (AVS) (2), left ventricular free wall (LV) (3) and the apex (4). In these regions the pattern of expression of the mRNA of the epicardial markers Raldh1, Raldh2, Wt1 and Tbx18 was established. Raldh1, Wt1 and Tbx18 are expressed in epicardial cells (Epi) covering the atria (b,e,j) and the atrioventricular junction (c,f,k). In the epicardial cells covering the apex Raldh1 (d) Wt1 (g) and are expressed, while Tbx18 (l) is not (*). In the epicardium covering the free wall none of these markers are expressed, as in examples Wt1 (h) and Raldh1 (i) is shown (*). Panels m-p show immunohistochemical staining for the progenitors markers PDGFRα (n) in cells present in the atrioventricular sulcus, CD34 (o) in the epicardium covering the apex, and c-Kit (p) in the epicardial cells covering the atria. Panel q provides a summary of the pattern of expression of the embryonic epicardial and progenitor markers in epicardial cells overlying the different areas of the heart. +* indicates that this marker is also present in the mesenchyme.
after left anterior descendent coronary artery (LAD) ligation. The infarcted area in these hearts was detected by the loss of expression of the mRNA encoding cardiac Troponin I (cTnI) as shown by in situ hybridization. Already one day after induction of myocardial ischemia, cTnI mRNA had disappeared from the ischemic area, albeit the protein is still detectable till three days post-MI. At all later time points neither mRNA nor protein can be detected in the infarcted region (figure 3).

Expression of the embryonic epicardial markers became more prominent after injury in the regions that expressed these genes in the control situation. Apart from these regions, expression of these mRNAs was highly increased in the epicardium covering the border zone of the infarcted area, one day post-MI (figure 3). From three days post-MI onwards the expression of mRNA of all embryonic epicardial markers was found in the newly formed epicardial cells covering the infarcted myocardium (figure 3). At this time point, Wt1 and Raldh1 and 2 are also expressed in the subepicardial mesenchyme, albeit at a lower level than in the epicardial cells. This pattern of expression persists up to two weeks post-MI. At one month post-MI, expression of Wt1, Tbx18, Raldh1 and 2 had largely become restricted to the epicardial areas that express these markers in the healthy heart. Their expression, however, remained in the border zone of the infarcted area but was no longer detected at three month post-MI.

**Activation of cardiac progenitor markers upon injury**

To establish whether the expression of cardiac progenitor marker genes, cKit, CD34 and PDGRFα, parallels the above-described changes in the newly formed epicardium, the patterns of expression were immunohistochemically determined at different time points after myocardial infarction (Figure 3). cKit became expressed in a portion of the epicardial cells that cover the border zone and ischemic myocardium. From one week post-MI onwards the expression of cKit, CD34 and PDGRFα was largely present in the subepicardial space overlying the injured myocardium (figure 3). Epi- and subepicardial cells of the the apex and atrio-ventricular sulcus also expressed these markers. Along with the down-regulation of embryonic epicardial genes two weeks post-MI, the expression of the markers for cardiac progenitor disappeared as well. Note that the patterns of expression of the cardiac progenitor marker genes parallel the patterns of expression of embryonic epicardial genes upon myocardial infarction, but that they are only expressed in a subset of the epicardial cells only.

**Epicardial and subepicardial proliferation**

Upon amputation of the apex in zebrafish, the epicardium is activated and myocardial cells located in the subepicardial layer start to proliferate. To investigate the initiation of proliferation in the mammalian heart upon myocardial infarction we examined the proliferation by analyzing the incorporation of BrdU. In control hearts, as expected, hardly any BrdU-positive cells were observed. As a control the gut was isolated and analyzed for
Figure 3 van Wijk et al

1. 1d post-MI
2. 3d post-MI
3. 1w post-MI
4. 1w post-MI

Legend:
- Progenitor marker
- cardiac Troponin I
- Nucleus
- 100µm
BrdU incorporation in parallel (figure 4). In the intestinal crypts, large numbers of BrdU-positive cells were observed, indicating that the mice were properly labeled. One day post-MI, extensive BrdU-incorporation was observed in the epicardium covering the atria and in some endocardial cells. During the following two weeks post-MI, BrdU was also detected in the epicardium covering the subepicardial space overlying the infarcted myocardium. Epicardial cells that cover the uninjured myocardium did not proliferate. From two weeks post-MI onwards, hardly any BrdU incorporation was observed in any part of the heart, except for some individual subepicardial cells in the border zone of the infarct. Such individual cells were still found at three month post-MI. BrdU incorporation was found to parallel the spatio-temporal changes in the expression pattern of the epicardial and progenitor cell markers, suggesting that epicardial and subepicardial cells transiently reactivate the expression of the embryonic gene program and proliferate upon myocardial infarction.

Subepicardial mesenchyme formation by EMT

The epicardium covering the atria and ventricles is in direct contact with the underlying myocardium in control hearts. In contrast, upon myocardial infarction the newly formed epicardium and ischemic myocardium became separated by extracellular matrix and subepicardial cells. As observed in our lineage analyses, subepicardial accumulations of mesenchyme were first observed in the border zone of the infarction, three days post-MI. The entire infarcted area was covered with subepicardial mesenchymal cells, one week post-MI. The formation of mesenchymal cells underneath the epicardium, that expresses embryonic epicardial genes is reminiscent to the capacity of the embryonic epicardium to undergo EMT and form cardiac progenitors. This prompted us evaluate the expression of markers of EMT, like Snai1 and α-sma. Three days post-MI, a layer of non-myocardial, Snai1 and α-sma
Figure 4. Proliferation of epicardial and subepicardial cells upon myocardial ischemia

In control hearts BrdU was not detected in the epicardium covering the atrioventricular junction (a), left ventricle (b) and atrium (c). As a positive control, BrdU incorporation was checked in the crypts of the gut in each mouse (d). At 2 days post-MI BrdU was detected in Wt1 positive epicardial cells (e), at 3 days post-MI in the epicardium covering the infarcted area (f) and atria (g) and in mesenchymal cell in the border zone (h). At one week post-MI BrdU was detected in cells located in the subepicardial space covering the infarcted area (i,j). At two weeks post-MI, BrdU was detected in a few individual subepicardial cells of the atria (k) and of the border zone of the infarct (l). At one month and three month post-MI hardly any BrdU incorporation was detected in the heart (m-p).
Figure 5. Post-ischemic mesenchyme formation
Snai1 mRNA is present from three days onwards in the subepicardial mesenchyme of the apex (a), and border zone (b). Raldh1 is expressed in epicardial cells and at a lower level in the subepicardial mesenchyme (c). In contrast to control hearts (d), α-sma is present in the subepicardial mesenchyme and in mesenchymal cells flanking myocardial cells at the epicardial side at one (e,f) and two weeks (g) post-MI. In these mesenchymal cells P-Erk is detected (i,j), while P-Smad is only found in subepicardial mesenchymal cells (arrow) directly flanking myocardial cells (k). Expression of P-Erk in control hearts (h). Within the border zone and infarcted area Follistatin-like 1 and Periostin are expressed in the non-myocardial cells (l-q). The scale bar indicates 100µm.
positive cells was present in the subepicardial layer on top of the border zone and infarcted area (figure 5). The presence of the expression of these markers in the subepicardial layer paralleled the expression of embryonic epicardial cells and the presence of mesenchymal cells in the subepicardial space between three days and two weeks post-MI. After two weeks only a few individual mesenchymal cells were observed to express these markers in the border zone of the infarction.

**Regulators of mesenchyme differentiation**

In cardiac regeneration studies, FGF-signaling was shown to be involved in the regenerative response mediated by the epicardium. Recently, we demonstrated that during development Smad-mediated BMP-signaling and Erk-mediated FGF-signaling cooperate in the regulation of the differentiation of progenitor cells into myocardial or epicardial cells. To establish whether these signaling pathways are also operational in the mesenchymal cells formed in response to injury, we determined the patterns of expression of activated Smad (P-Smad) and Erk (P-Erk). P-Smad was neither detected in epicardial nor subepicardial cells in control or infarcted hearts, whereas P-Smad expression was detected at low, though significant, levels in the myocardial cells directly flanking the subepicardial mesenchymal layer. P-Erk, on the other hand, was observed in a small portion of epicardial cells in control hearts (figure 5), but was strongly up-regulated within the first week post-MI in the newly formed mesenchymal cells present in the subepicardial space (figure 5). Along with the upregulation of embryonic epicardial progenitor cells and EMT markers, P-Erk was upregulated from three days onward until two weeks post-MI. At 1 month post-MI, P-Erk was detected only in the subepicardial mesenchyme of the border zone of the infarcted area.

Matricellular proteins belong to a family of proteins that affect differentiation, migration, matrix production and maturation of cells. These proteins function by their ability to interact with multiple cell surface receptors, especially integrins, cytokines, growth factors, and proteases, but also by direct binding to structural or scaffold proteins. The expression of this family of proteins is most prominent during development but is also shown to play an important role in the response to injury. We investigated the expression of two members of the matricellular protein family, Follistatin-like-1 (Fstl1) and periostin (figure 5). Fstl1 and Periostin were both present in the entire infarcted area, with Fstl1 being more extensively expressed than Periostin. Both matricellular proteins were most abundantly present at one week post-MI, after which their expression gradually reduced to very low but still detectable levels at two weeks post-MI. After one and three month post-MI their expression was not detected.
Discussion

Our study shows that the epicardium functions as a transient source of mesenchymal cells in the endogenous regenerative response of the mammalian heart. These mesenchymal cells differentiate into cardiomyocytes, myofibroblasts and endothelial cells. The epicardial response to injury is activated within one day post-MI and is present till two weeks post-MI. Cardiomyocytes formed by this process are detected first at one month post-MI. Within the epicardial regenerative response three phases can be distinguished (1) the expression of embryonic epicardial genes and markers of progenitor cells, (2) the initiation of local epicardial and subepicardial proliferation and (3) the formation of mesenchyme (figure 6). An important finding of our study is that the mammalian heart displays an endogenous regenerative response comparable to that of lower vertebrates. Although the mammalian heart is not capable to regenerate the lost myocardium, a limited number of myocardial cells is formed from epicardial derived cells which are formed during a short window of time in the infarcted area.

The epicardium is the source of newly formed cardiomyocytes upon injury

The Wt1 lineage reporter we used to analyze the contribution of Wt1-expressing cells to the infarcted heart does not allow inducible activity of Cre, which would enable us to visualize the lineage positive cells without their developmental history. Nonetheless, we can visualize the post-ischemic contribution of Wt1-expressing cells to the infarcted area because we analyzed the time dependent expression of β-Gal after a myocardial infarction and compared the changes in the pattern of β-Gal expression to uninjured areas of the same heart and control hearts. The observation that the epicardium, which was lost from the infarcted area within one day upon MI, recovered in three days with β-Gal positive epicardial cells, suggests that these cells take origin from the remaining epicardium at the border zone. This conclusion is underscored by the time-dependent formation of a β-Gal-positive subepicardial mesenchymal layer and differentiated cells in the infarcted area.

Pro-fibrotic mesenchymal signaling inhibits cardiomyocyte formation

Subsequent to myocardial infarction, mesenchymal cells are formed from the epicardium by EMT and proliferation within a relatively short window of time. In this mesenchyme, increased expression of important regulators of mesenchymal differentiation and migration, like periostin and follistatin-like1, both members of the matricellular protein family, was observed (Figure 5). Follistatin-like1, a potential natural BMP inhibitor, has been suggested to serve a protective role after ischemia, as adenoviral delivery of Fstl1 to ischemic myocardium reduced the size of the myocardial infarction,28 and promoted revascularization after hind-limb ischemia.29 Periostin, is described to be an important regulator of differentiation of mesenchymal cells into fibroblasts.30-32 Upon MI in mice, in which periostin was specifically deleted, more myocardial cells were formed at the expense of fibroblasts, indicating that
Figure 6. Schematic overview of morphological and molecular changes upon myocardial ischemia
periostin is a negative regulator of myocardial differentiation and functions as a pro-fibroblastic factor. In line with this function of periostin, we observed formation of only a few cardiomyocytes as assessed by the expression β-Gal.

Activated Erk-signaling, present in the mesenchyme formed upon cardiac injury, is described to promote non-myocardial differentiation. During development, Erk-mediated FGF-signaling has been shown to inhibit pro-myocardial Smad-mediated BMP-signaling of cardiac progenitor cells. The observed high abundance of P-Erk and low abundance of P-Smad in the subepicardial mesenchyme formed upon MI suggests that a similar cooperative interaction between BMP- and FGF-signaling is operational in the adult heart as in the embryonic heart. This might be responsible for the observed pro-fibrotic response.

**Regeneration as a recapitulation of epicardial development**

Developmental studies suggest that the epicardium might function as a progenitor pool for the heart. A recent analysis of the function of Wt1 in the epicardium during development, suggested that Wt1 is important in the formation of cardiac progenitor cells. Such a function is in line with the observation that the epicardium serves as a multi-potent layer of cells contributing to valvular mesenchyme, cardiac fibroblasts, smooth muscle and endothelial cells of the coronaries during development. Recent genetic lineage analyses, using mice in which Cre was inserted into the translation start site of Wt1, identified not only non-myocardial cells, but also a small amount of cardiomyocytes to be derived from the epicardium. Moreover, during development, the epicardium secretes growth factors, like Fibroblast Growth Factor, Bone Morphogenetic Proteins and Retinoic Acid that stimulate the underlying myocardium to proliferate, thereby forming the ventricular compact layer. Along with these paracrine signals, epicardial-derived fibroblasts influence proliferation of myocardial cells in the compact layer by direct cellular interaction.

During life and upon injury, new cardiomyocytes are formed. The source of these newly formed cardiomyocytes is unknown. From a developmental stance, the adult heart comprises cells that originate from three major lineages, the endocardial, myocardial and epicardial lineage. Genetic lineage tracing has shown that these newly formed cardiomyocytes are not derived from the myocardial component of the adult heart. Zeisberg and colleagues showed in an endocardial lineage analysis (Tie1Cre) that in response to injury the endocardium only contributes fibroblasts and not cardiomyocytes. In this study we show in a epicardial lineage analysis that the myocardial cells that are found in a myocardial infarction, are mainly derived from Wt1-expressing epicardial cells. The observation that a new layer of epicardium is formed over the ischemic area and contributes mesenchymal cells to replace the dead cardiomyocytes, suggests that cardiomyocytes found in the infarct originated from the overlying epicardium.
Evolutionary conservation of the endogenous cardiac regenerative response

At first glance, the zebrafish regenerative response seems to be entirely different from the regenerative response described in the mouse. In this study, however, we show extensive similarities especially in the initial responses upon injury. As in zebrafish, the epicardium is activated, both in the border zone and later in the epicardium covering the infarcted area as well in more remote epicardium covering the atrioventricular sulcus and atrium. However, analyzing the extent of proliferation in the infarcted mouse heart, differences were observed compared to zebrafish. Although it was originally suggested that the newly formed myocardial cells originate from the epicardium, recent studies pointed to proliferation of myocardial cells.5,6 Our data indicate that in mice the newly formed cardiomyocytes are derived from the epicardium, as originally suggested in zebrafish.7 This is further underscored by the finding that newly formed myocardial cells upon a myocardial infarction are not derived from existing cardiomyocytes using an inducible myocardial Cre-expression analyses in mice.4

Taken together, this analysis shows that a regenerative response, comparable to the response observed in fish, is also initiated in the mouse. This reveals that an evolutionary conserved mechanism also appears to persist in the mammalian heart. However, unlike in fish, the regenerative response of the higher vertebrate, mammalian heart is abrogated, resulting in the formation of only a limited number of new cardiomyocytes. These findings provide a level of basic knowledge which could be useful for the development of new strategies to enhance the endogenous regenerative response of the myocardium and eventually heal the infarcted heart.

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